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Surface coating of PLGA microparticles with protamine enhances their immunological performance through facilitated phagocytosis

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Abstract

Surface modifications of poly(lactide-co-glycolide) microparticles with different polycationic electrolytes have mainly been studied for conjugation to antigens and/or adjuvants. However, the *in vivo* immunological effects of using surface charged particles have not been address yet. In this study, microparticles were coated or not with protamine, a cationic and arginine-rich electrolyte that confers microparticles with a positively surface charge. We then evaluated the potential of protamine-coatings to assist the induction of immune responses in mice. Interestingly, enhanced antibodies and T-cell responses were observed in mice treated with the coated particles. *In vitro* studies suggested that the improved immunological performance was mediated by an increased uptake. Indeed, protamine-coated particles that carried a plasmid were even internalised into non-phagocytic cells and to cause their transfection. These results open the way for further research into a novel technology that combines the use

protamine for facilitated cell penetration of that and biodegradable microparticles for prolonged antigen or drug release.

Keywords: Microparticles; Protamine; Transfection; Uptake; Immune response

Article Outline

1. Introduction

Polymeric biodegradable microparticles have been widely studied for a broad variety of pharmaceutical and biomedical applications [1], [2] and [3]. Commonly used poly(lactide-co-glycolide) (PLGA) types are available in medical grade and approved for use in humans, which makes them attractive for developing new drug and antigen delivery systems [4], [5] and [6]. With a size range similar to that of microorganisms, polymeric particles can be easily taken up by antigen-presenting cells (APCs) [7], a process that enables potent cellular as well as humoral immune responses [8], [9], [10], [11] and [12].

Physico-chemical properties such as the molecular weight and monomer composition of the polymer, and the size and surface charge of the microparticles determine the antigen release rate [12] and may also affect the type of immune response elicited by the delivery system [6] and [13]. To add further flexibility and control of antigen and adjuvant delivery, the surface properties of PLGA microparticles have been modified [14], [15] and [16]. Surface modifications have been achieved with anionic electrolytes such as sodium dioctylsulfosuccinate [17] and [18] and cationic electrolytes such as chitosan, poly(ethylene imine), or protamine [14], [15], [16] and [19]. Protamine belongs to a group of low molecular weight (MW: 4000–4250 Da), arginine-rich, basic proteins, which condense DNA in the nucleus and are involved in spermatogenesis [20]. Protamine is a FDA-approved compound, which has found applications in, e.g., stabilising DNA [21], insulin complexation and formulation, and in reverting the anticoagulant effect of heparin [22], [23] and [24]. Protamine has been combined into PLGA microparticles [14] and [26] and used as a coating agent to bind nucleic acid on the surface of controlled-release particles [27], and on one occasion, the use of protamine-containing PLGA microparticles in mice is reported [25].

The aim of this study was to evaluate the properties of protamine-coated PLGA microparticles with regard to particle uptake in cells, transfection of cells, antigen presentation, and the induction of T-cell and antibody responses in mice. Interestingly, the protamine coating enhanced particle uptake even by non-phagocytic cells and promoted the stimulation of much stronger immune responses as compared to uncoated particles.

2. Materials and methods

2.1. Materials

Purified phospholipase A_2 (PLA2) from bee venom and chicken egg albumin (OVA; grade V) were purchased from Sigma-Aldrich (Buchs, Switzerland), and soy bean lecithin (Epikuron 200) from Degussa (Hamburg, Germany). Phosphorothioate-modified CpG oligodeoxynucleotide 1668 pt (5′-TCC-ATG-ACG-TTC-CCT-GAC-GTT-3′) was synthesised by Microsynth (Balgach, Switzerland). The 35 kDa poly(lactide-co-

glycolide) (PLGA 50:50) with uncapped end-groups (Resomer RG503H) was from Boehringer-Ingelheim (Ingelheim, Germany). Protamine sulphate from salmon, poly(vinyl alcohol) (PVA; Mowiol 4-88) and dichloromethane (DCM) were from Fluka (Buchs, Switzerland). The GFP plasmid (pmaxFP-Green-C) was provided by Amaxa (Cologne, Germany).

2.2. Mice

BALB/c, CBA/J and C57BL/6 mice were purchased from Harlan (Horst, The Netherlands) and used at 6–10 weeks of age. DO11.10 mice were generously provided by Martin F. Bachmann (Cytos Biotechnology, Switzerland) and are transgenic for the T-cell receptor recognising the $OVA_{323–339}$ epitope on a $2H^d$ MHC-class II context in a BALB/c background [29]. OT-II mice were generously provided by Tobias Suter (University of Zurich, Switzerland). Also these mice are transgenic for the T-cell receptor recognising the $OVA_{323–339}$ epitope but on a C57BL/6 ($2H^b$ MHC II) background [30]. All mice experiments were approved and performed according to guidelines formulated by the Veterinary authorities of the canton of Zurich, and the mice were kept in a specific pathogen free (SPF) environment.

2.3. Microparticle preparation

PLGA microparticles were made by microextrusion-based w/o/w-solvent extraction using a static multilamination type micromixer (Institut für Mikrotechnik Mainz, Mainz, Germany), as previously described [28], with slight modifications. Three groups of formulations with different loads were prepared, with and without a protamine coating on the surface (Table 1). In the first group of microparticles, PLA2 was encapsulated for later evaluation of humoral responses. Briefly, 2 mg of PLA2 was dissolved in 100 µl water and emulsified on ice (ultrasonication with a probe for 10 s; 40% amplitude, UP200H, Hielscher Ultrasonics, Teltow, Germany) into 0.6 g of PLGA dissolved in DCM (5%, w/w). This w/o-emulsion was extruded through the micromixer along with a 0.5% (w/w) PVA aqueous solution as extraction phase; the resulting suspension of microparticles was collected using the same aqueous solution of 0.5% (w/w) PVA for further solvent extraction and evaporation. To obtain positively charged microparticle coating, 0.5% (w/w) protamine solution was used as first extraction phase and water as

final collection fluid. The particles were gently stirred using a magnetic rod and kept under laminar air-flow for 30 min for further solvent removal and hardening. Finally, the microparticles were collected on a mixed cellulose ester membrane filter with a pore size of 0.8 μm (Whatman, Dassel, Germany) and dried at 20 mbar and at room temperature for 24 h.

Table 1.

Microparticle formulations

Test formulation	**Zeta potential (mV)**	**Size distribution (μm) (D_{10}/D_{50}/D_{90})**	**Antigen loading (μg/mg MP)**	**Study application**
pGFP-MP	− 11.8 ± 0.8	1.6/3.2/5.2	0.76	Particle uptake and transfection
pGFP-MP/protamine	64.8 ± 4.3	1.9/3.5/5.3	0.76	
OVA-MP	− 11.6 ± 0.1	1.4/3.2/6.4	37.9	Antigen presentation and T cell response
OVA-MP/protamine	19.3 ± 0.6	2.3/7.2/20.9	37.9	
PLA2-MP	− 2.4 ± 4.2	1.2/4.4/24.9	0.84	B-cell responses
PLA2-MP/protamine	10.5 ± 6.6	3.0/6.8/15.5	0.77	

Characteristics and application of the different groups of formulations used in the current the study.

The second group of microparticles, with and without protamine coating, contained pGFP. They were prepared for analysing particle uptake by cells and particle-mediated cell transfection. Briefly, pGFP (0.7 mg) was dissolved in Tris–EDTA (TE) buffer (0.2 ml) and emulsified in PLGA (0.2 g) dissolved in DCM (5%, w/w). The subsequent steps for microparticle formation were as described above.

The third group of microparticles, with and without protamine coating, was loaded with OVA for subsequently analyses of T-cell responses. Briefly, OVA (50 mg) was dissolved in PBS (0.8 ml), emulsified in PLGA (1 g) dissolved in DCM (5%, w/w), and further processed as described above.

2.4. Particle size and zeta potential measurements

Microparticle size distributions were determined by laser light diffraction (Mastersizer X, Malvern Instruments Malvern, United Kingdom) with a suspension of approx. 1 mg of dry microparticles in distilled water. A volume-weighted size distribution with 10%, 50% and 90% undersize diameters (D_{10}, D_{50} and D_{90}) was determined. The surface charge of the microparticles was determined by zeta potential measurement (Zetasizer 3000 HAS, Malvern Instruments). Dry microparticles (0.2 mg) were suspended in 1 mM KCl (2 ml; pH = 7.6), and the mean zeta potential value was calculated from two samples per batch and triplicate analysis per sample.

2.5. Determination of antigen content in the microparticles

Triplicates of approx. 7 mg PLA2- or pGFP-containing particles were dissolved in 0.5 ml chloroform and the protein or the plasmid extracted from the organic phase by mixing with 0.2 ml Tris–HCl buffer and subsequent centrifugation at 3000 rpm for 3 min. The content of plasmid DNA from the pGFP-MPs was analysed by the PicoGreen quantification kit (Invitrogen AG, Switzerland) following the manufacturer instructions. The PLA2 concentration in the aqueous extract was analysed by an ELISA inhibition assay. Briefly, 60 µl of the Tris–HCl phase containing the extracted PLA2 was mixed in a 96-well plate with 60 µl human anti-PLA2 serum diluted (1:25) in phosphate-buffered saline containing 0.05% Tween20 and 2.5% skimmed dry milk (PBSTM). In parallel, serial dilutions of PLA2 were mixed with sensitised serum and utilised as a standard for quantification. After 2 h of incubation at 37 °C, 100 µl of each sample was transferred to an ELISA plate that was pre-coated with 5 µg/ml PLA2 at 4 °C overnight and subsequently blocked with PBSTM at room temperature for 1 h. After 2 h incubation at 37 °C, the plate was washed and incubated with horseradish peroxidase-conjugated anti-human IgG (BD Biosciences Pharmingen, San Diego, CA). The plate was washed and developed with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium

salt (Sigma-Aldrich) in 1 M sodium dihydrogen phosphate and the absorption was read at 405 nm using a plate reader. The loading efficiency of PLA2 in the MP was calculated relative to the theoretical maximum loading value. For the quantification of OVA inside the MPs, OVA-containing particles were dissolved in 0.1 M NaOH and 5% SDS overnight at room temperature and then measured by the BCA protein assay (Pierce Biotechnology, USA).

2.6. Internalisation of pGFP-loaded PLGA microparticles (MP) by HEK cells

The role of the protamine in the particle uptake by cells was studied by measuring the transfection of Human Embryonic Kidney (HEK) cells upon incubation with microparticles containing the plasmid pGFP, taking use of the fact that positive transfection would necessarily imply uptake of the plasmid containing particle by the cell; preliminary studies with FITC or coumarin-loaded particles were also done, but this did not allow to clearly distinguish between internalised particle and particle attached to the cell surface. Cells were seeded in 24-well plates pre-coated with poly-d-lysine at a density of 3×10^5 per well. One day after seeding, 50, 100 or 200 µg of pGFP-MP or pGFP-MP/protamine were added to the cells in duplicates; after incubation for 4 h and various subsequent washes, the cells were incubated with fresh complete medium. The transfection of cells was monitored for two further weeks using a Zeiss Axiovert fluorescence microscope (Carl Zeiss, Jena, Germany). The average number of transfected (green) cells in 10 randomly selected bright light fields was counted.

2.7. *In vitro* analysis of T-cell stimulation

Murine dendritic cells were isolated from BALB/c bone-marrow and grown in 10 cm Petri dishes at a density of 1×10^6 cell/ml in 10 ml RPMI-1640 complete media containing 20 ng/ml of granulocyte macrophage colony stimulating factor (GM-CSF) and 10 ng/ml of IL-4 (Immunotools, Friesoythe, Germany). After 2 and 4 days, the culture medium was replaced by fresh medium containing GM-CSF and IL-4. After 6 days, immature DCs were collected and seeded at 1×10^5 cells per well in a 96-well plate and then pulsed for 3 h at 37 °C and 5% CO_2 with escalating amounts of OVA-containing microparticles, empty microparticles (1 or 100 µg) or aqueous OVA (10 or 100 µg/ml). After three washing steps, the cells were co-cultured with splenocytes (1×10^5

cells/well) from DO11.10 mice. After 20 and 48 h, aliquots of the supernatant were taken for determination of IL-2 and IFN-γ secretion, respectively. Lymphocyte proliferation was measured after 48 h by [^{3}H] thymidine (1 μCi/well) incorporation during the last 16 h of culture using a 1450-MicroBeta TriLux liquid scintillation counter (Wallac, Finnland).

2.8. Immunisation of mice

To evaluate the potential of the microparticles to stimulate T-cell responses *in vivo*, 6–10 week old female OT-II mice were used. The mice were injected with 1 mg of OVA-MP or similar particles coated with protamine (OVA-MP/protamine); for injection, the microparticles were suspended in 1% aqueous lecithin solution. Blood was withdrawn at different time points (7, 14 and 21 days), and the lymphocytes analysed by flow cytometry for IFN-γ producing cells.

For the study of antibody responses, PLA2-containing microparticle formulations were tested in 6–10 weeks old female CBA/J mice. The formulations PLA2-MP or PLA2-MP/protamine were suspended in 1% aqueous lecithin solution prior to subcutaneous injection of a particle dose equivalent to 1 μg of entrapped PLA2; 1.2–1.3 mg of microparticles were injected in a volume of 150 μl. The mice were boosted with the same preparation and dose after 28 days. Serum was prepared from blood taken on days 28, 55 and 84 and frozen at − 20 °C until analysed by ELISA. Additional two groups of mice were injected with the same two formulations PLA2-MP or PLA2-MP/protamine after addition of 3.1 nmol CpG.

2.9. Flow cytometry

Intracellular IFN-γ was assessed in blood of the OT-II mice injected with 1 mg of OVA-MP or OVA-MP/protamine. After lysis of the erythrocytes with hypotonic Red Blood Cell Lysis Buffer (Sigma-Aldrich), splenocytes were stimulated *in vitro* with soluble OVA (10 μg/ml) and brefeldin A (10 μg/ml) (Sigma-Aldrich) for 3 h at 37 °C and 5% CO_2. For FACS staining, the cells were washed several times, incubated with anti-CD16/CD32 for Fc-receptor blocking (5 min), and stained with anti-CD44-FITC and anti-CD4-APC antibodies for 30 min. Cells were then fixed with 1% paraformaldehyde for 10 min and

subsequently stained with anti-IFN-γ-PE in PBS/0.5% saponin for 30 min. All stainings were done in PBS/2% FCS at 4 °C, unless specified otherwise, and all antibodies were purchased from BD Pharmingen (Franklin Lakes, NJ, USA). Samples were measured on a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA), and data was analysed using FlowJo software (Ashland, OR, USA).

2.10. Antibody determination by enzyme-linked immunosorbent assay (ELISA)

For detection of PLA2 antibodies, microtitre 96-well plates (Nunc Maxisorb, Wiesbaden, Germany) were coated with 5 µg/ml PLA2 in carbonate buffer at 4 °C overnight. The plates were blocked with 2.5% skinned dry milk in phosphate-buffered saline containing 0.05% Tween 20 (Serva, Heidelberg, Germany) (PBSTM) for 1 h. Then, serial dilutions of individual mice sera in PBSTM were incubated in the plates for 2 h. Next, the plates were incubated with 1 µg/ml biotinylated goat anti-mouse IgG1 or IgG2a (BD Bioscience Pharmingen) in PBSTM for 2 h. Subsequently, plates were incubated with a 1:1000 dilution of streptavidin-conjugated horseradish peroxidase (BD Pharmingen) for 1 h and developed with the enzyme substrate 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (Sigma-Aldrich) in 1 M sodium dihydrogenphosphate. The endpoint optical density was measured at 405 nm after 20 min incubation using a microplate reader Model 550 (BioRad, Hercules, CA, USA). All steps were intercepted by washings with PBST.

3. Results

3.1. Microparticle characterisation

Three different groups of microparticles, all with and without protamine coating, were prepared (Table 1). The formulations with encapsulated pGFP, named pGFP-MP or pGFP-MP/protamine, had a very similar size distribution. Fifty percent of the particles were smaller than 3.2 µm or 3.5 µm, respectively. For both OVA- and PLA2-containing formulations, the size of the protamine-coated particles was larger than that of the non-coated particles. This was most evident for the OVA-containing microparticles, where 50% of the OVA-MP/protamine were smaller than 7.2 µm as compared to 3.2 µm for the non-coated particles (Table 1). Independent of the encapsulated antigen, the zeta

potential of the protamine-coated microparticles was, as expected, positive (+ 11 to + 65 mV), which is due to the polycationic charge of protamine; on the contrary, the non-coated particles had negative zeta potential (ranging from − 11.8 to − 2.4 mV).

3.2. *In vitro* internalisation of pGFP-loaded microparticles

To examine the effect of the protamine coating on the internalisation of the microparticles, HEK cells were incubated for 4 h with different amounts of pGFP-containing particles, with or without protamine coating. Transfection of the cells was assessed as a measure for particle internalisation by fluorescence microscopy. As illustrated in Fig. 1A, only protamine-coated particles were able to transfect HEK cells. The transfection was dose-dependent, as the efficacy increased with the amount of particles used in the culture (Fig. 1B) and the frequency of transfected cells was approx. 0.4% (0.1–1.0%).

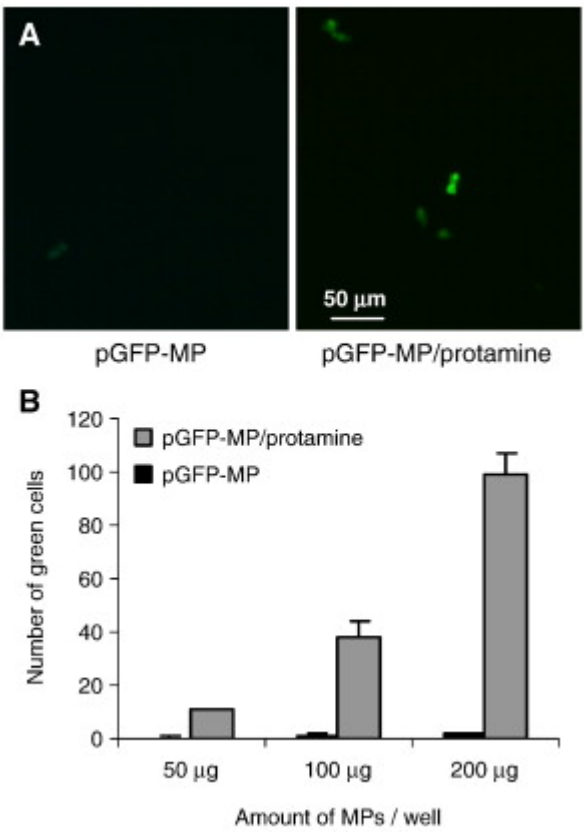

Fig. 1. Internalisation of microparticles containing pGFP by HEK cells. (A) Fluorescence microscopy pictures of cells incubated with 100 μg of protamine-coated or uncoated microparticles. (B) Quantification of positive cells as assessed by counting the green cells in 10 bright randomly selected light fields per well, after 72 h of incubation with the microparticles. The data is representative of three similar experiments.

3.3. *In vitro* proliferation of specific T cells

To assess the role of the protamine coating in the presentation of encapsulated antigen, OVA-containing microparticles, with and without protamine coating, were incubated at escalating amounts with bone-marrow derived DCs from BALB/c mice. The pulsed DCs were then co-cultured with splenocytes derived from transgenic DO11.10 mice. After 20 h, the secretion of IL-2 in the supernatant was measured by ELISA and after 48 h proliferation was determined by measuring the [^{3}H] thymidine incorporation. Fig. 2A reveals that the protamine coating enhanced the IL-2 secretion in a dose-dependent manner and with a 10-fold higher sensitivity than the uncoated particles. Correspondingly, the protamine-coated particles also showed a 10-fold increased capacity to stimulate the secretion of IFN-γ (Fig. 2B) as well as the proliferation of antigen-specific T cells (Fig. 2C). This effect of protamine was also observed when compared to stimulation of splenocytes using DCs pulsed with soluble OVA. Cells stimulated with 100 μg/ml or 10 μg/ml OVA solution produced very low or even undetectable levels of IL-2, and moderate and concentration-dependent T-cell proliferation. Unloaded microparticles, with or without the protamine coating, did neither stimulate IL-2 production nor lymphocyte proliferation (data not shown).

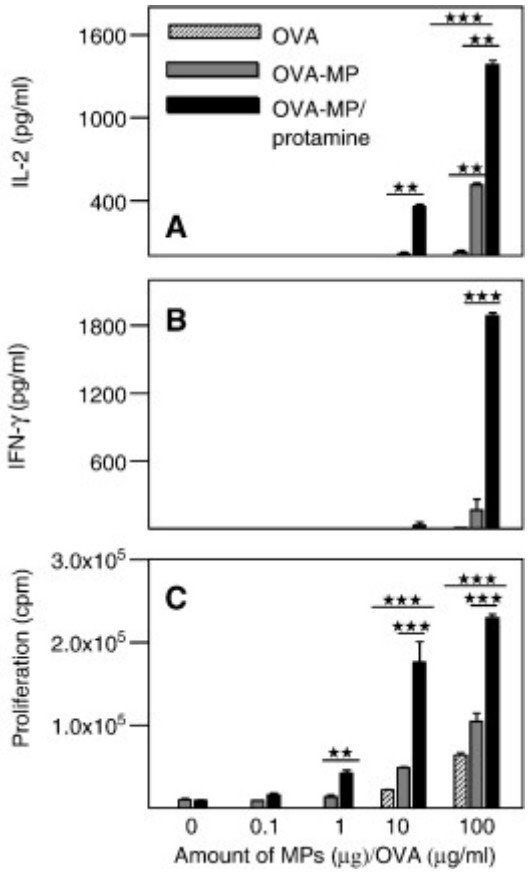

Fig. 2. *In vitro* stimulation of OVA specific T cells. Bone-marrow derived DCs from BALB/c mice were pulsed for 3 h with different amounts of OVA-loaded microparticles and then incubated with splenocytes from DO11.10 mice in tripiclates. After 20 h, IL-2 (A), and after 48 h, IFN-γ (B) was measured in the supernatants, and cell proliferation was determined by [^{3}H] thymidine incorporation (C). Data was analysed by Two Way ANOVA with Bonferroni post test (***$p < 0.0001$; **$p < 0.01$).

3.4. Stimulation of T-cell responses in mice

The capacity of microparticles to enhance the induction of T-cell responses *in vivo* was evaluated in transgenic OT-II mice. Animals were injected with 1 mg of either uncoated or protamine-coated microparticles containing OVA protein, OVA-MP or OVA-MP/protamine, and T-cell responses were assessed by measuring the activated CD4 T cells producing IFN-γ after 7, 14 and 21 days. Mice injected with OVA-MP/protamine

induced higher frequencies of IFN-γ producing CD4 T cells at all time points as compared to mice that received OVA-MP (Fig. 3). As early as day 7 (Fig. 3A) the protamine-coated microparticles induced significant levels of IFN-γ producing cells, in contrast to the uncoated microparticles that needed an additional week to raise a moderate response. At day 14, less than 2% of the cells from the mice immunised with the uncoated particles were IFN-γ producing CD4 T cells as compared to 3% achieved with the protamine-coated particles (Fig. 3B). Maximal response was attained with both formulations at day 14 after the injection (Fig. 3B). Untreated mice were used to determine the background levels of IFN-γ in the OT-II mice.

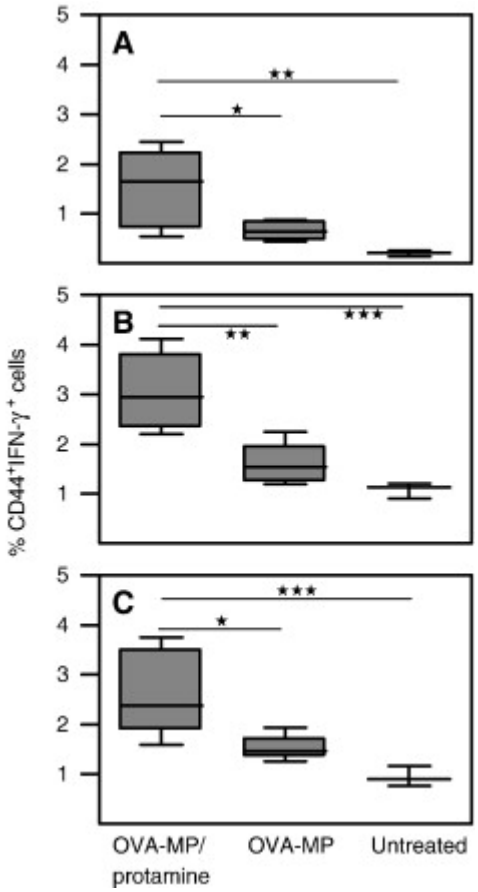

Fig. 3. *In vivo* induction of T-cell responses. OT-II mice were immunised with microparticles containing OVA (*n* = 5), and the percentage of IFN-γ producing CD4 T cells in peripheral blood was measured by flow cytometry after 7 (A), 14 (B) and 21 (C)

days. Untreated mice were used as negative controls. Data were analysed by One Way ANOVA with Tukey's Multiple Comparison Test (***$p < 0.0001$; **$p < 0.01$; *$p < 0.05$).

3.5. Stimulation of B-cell responses in mice

To evaluate the effect of the protamine coating on the stimulation of B-cell responses, CBA/J mice were immunised with microparticles containing the major bee venom allergen PLA2. The antibody responses were determined at 28, 55 and 84 days after the first injection. After the first injection, the protamine-coated particles induced a weak PLA2-specific IgG1 response, while the uncoated particles failed to do so, as measured 28 days after the priming (Fig. 4A). When a second dose was administered at day 28, the IgG1 response was strongly boosted by the protamine-coated particles, but not by the uncoated particles. The IgG1 levels remained high for at least 84 days. Two injections were required to induce a detectable IgG2a response; and again, the response, although weak, was stronger in mice immunised with the protamine-coated particles (Fig. 4B).

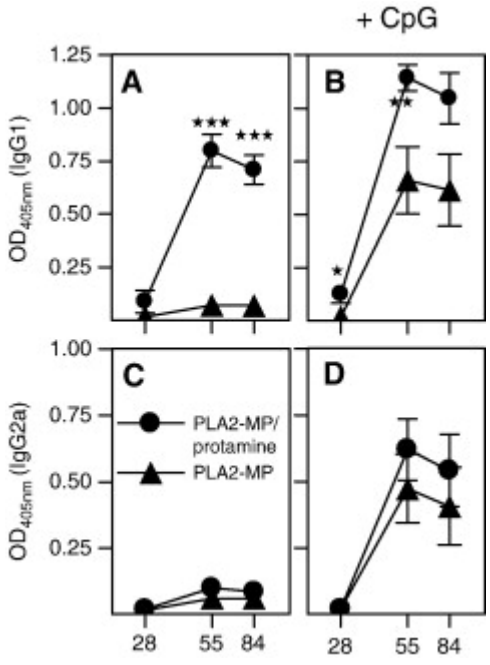

Fig. 4. *In vivo* stimulation of humoral responses. CBA/J mice were immunised at day 0 and boosted at day 28 with PLA2-containing microparticles, with or without protamine coating (A,B), and admixed with CpG (C,D) ($n = 4$). Blood was collected at days 28, 55 and 84, and analysed by ELISA for anti-PLA2 IgG1 (A,C) and IgG2a (B,C) antibodies. The absorbance at 1/800 serum dilution was chosen for comparing the groups at each time point. Data were analysed by One Way ANOVA with Tukey's Multiple Comparison Test (***$p < 0.0001$; **$p < 0.01$; *$p < 0.05$).

For assessing whether the observed benefit of the protamine coating persisted in the presence of an additional adjuvant, the two PLA2-loaded microparticle formulations were admixed with immune stimulatory CpG prior to injection. The previously observed Th2-biased response was shifted in the presence of CpG towards Th1, with both PLA2-MP and PLA2-MP/protamine (Fig. 4C and D). The IgG1 levels increased for both formulations, but the protamine-coated particles again stimulated stronger responses than the uncoated particles.

4. Discussion

Microparticles of PLGA have been widely studied for their use as drug and antigen delivery systems [3], [31] and [6]. These particulate systems are able to deliver drugs or antigens such as DNA, proteins, and peptides over prolonged periods of time, and induce strong immune responses against encapsulated antigens [13], which makes them attractive candidates for vaccine development.

Stability issues with encapsulated biologicals have led to an alternative method for associating antigens and DNA with PLGA microparticles, namely by adsorbing the bioactive materials onto the surface of anionic or cationic particles [16] and [18]. Yet, not many studies have addressed the question if and to what extent the modified particle surface itself influences the induction of immune responses *in vivo*. In the current study, we investigated the immunological effects of coating PLGA microparticles with the cationic electrolyte protamine for stimulation of humoral and cellular immune responses to various antigens.

In mice immunised with microparticles containing the bee venom major allergen PLA2, we found that protamine-coated particles were more immunogenic than uncoated particles and enhanced the induction of long-lasting IgG1 antibody responses. In mice, the IgG1 subclass is associated with Th2 responses and is important to fight extracellular pathogens. In contrast, IgG2a is associated with Th1 and cytotoxic T-cell responses that are essential for inflammatory processes and in controlling intracellular pathogens. Although the protamine-coated particles also stimulated stronger IgG2a antibody responses than the uncoated microparticles, the overall IgG2a/Th1 response was weak, but could be strongly improved by admixing immunostimulatory CpG; in the presence of CpG, protamine-coated microparticles promoted again a stronger immune response than the uncoated particles. This capacity of polarising the immune responses towards Th1 or Th2, as a function of the co-formulation with immune response modifiers, may be exploited for different vaccination strategies, depending on the desired outcome [32].

The improved humoral immune responses after immunisation with the protamine-coated microparticles prompted us to study whether protamine also affected the stimulation of T cells. Indeed, enhanced T-cell responses to ovalbumin were observed in mice after using OVA-containing particles coated with protamine. Although, differences were not as striking as for the humoral responses, there was a significant increase in the percentage of IFN-γ producing CD4 T cells at all time points examined when compared to the uncoated particles. The enhanced immunogenicity of the protamine-coated particles was mediated through their preferential interaction with professional antigen-presenting cells. Bone-marrow derived DCs pulsed with protamine-coated microparticles stimulated antigen-specific T cells *in vitro* more efficient than did DCs pulsed with uncoated microparticles.

One potential reason for the difference between protamine-coated and protamine-free particles with respect to their interaction with APCs could be differences in the particles size distribution of the formulations. In this study, the size distribution of the uncoated OVA-containing microparticles was ideal for uptake, since 90% of the particles were smaller than 6.4 μm, and only particles smaller than 5–10 μm have been shown to be efficiently taken up by antigen-presenting cells [7] and [33]. However, as the protamine-

coated microparticles had a broader size distribution than the uncoated ones, with only 50% of the particles being smaller than 7.2 µm, the size cannot explain the differences *in vitro* and *in vivo* immune responses.

Besides particle size, particle surface charge may also influence the interaction with APCs. Positively charged particles are more likely to bind to the negatively charged cell surfaces, and thereby facilitate uptake and stimulation of immune responses. Indeed, cationic microparticles have been reported to enhance phagocytosis by DCs and macrophages in comparison with unmodified particles [34], [35] and [36]. The mechanism suggested for the enhanced uptake in those studies is the electrostatic attraction between the positively charged microparticles and the negatively charged cell surface mediating binding and subsequent internalisation. In addition to its positive charge at physiological pH, protamine specifically contains arginine-rich sequences, which share structural similarity with certain viral proteins such as the Tat from the Human Immunodeficiency Virus (HIV-1) or the VP22 from the Herpes Simplex Virus, both of which possess protein translocation activity [37], [38] and [39]. These sequences of basic amino acids mediate the penetration of viruses into cells in an energy-independent manner [40]; thus, these so-called cell-penetrating peptides or peptidic moieties appear to be particularly good candidates as transporters for drug delivery. While the cell-penetrating property of Tat peptides have been extensively studied [41], [42] and [43], the potential of protamine to carry cargos across the cell membrane is less recognised. However, in 2005, two independent groups reported that protamine can act as an efficient membrane-translocating peptide both *in vitro* [44] and *in vivo* [44] and [45]. Park et al. showed that a protein toxin conjugated to protamine was able to translocate into cells *in vitro*, resulting in the inhibition of tumour growth *in vivo*. It was also reported that Tat-carrying [46] and protamine-carrying [44] iron nanoparticles were readily internalised by cells. Moreover, Tat-liposomes of 200 nm showed potential for intracellular gene delivery *in vitro* and *in vivo* [47]and [48]. These experiments suggest that the improved immunological performance of protamine-coated antigen-containing PLGA microparticles was mediated by the cell-penetrating properties of the arginine-rich protamine. We could confirm this by testing the transfection of bone-marrow DCs using microparticles containing a plasmid coding for GFP (data not shown). However, to test this hypothesis in a more stringent model, we also tested the uptake in and transfection

of non-phagocytic HEK cells. Indeed, protamine-coated particles were capable to transfect the HEK cells, whereas uncoated particles did not possess this capacity. The same was observed when testing the particles on non-phagocytic melanoma cells (no shown). As the size distribution of the tested formulations was almost identical (± 0.3 µm), the observed differences must be ascribed to protamine and its ability to translocate the microparticles intracellularly.

Although, there is certain controversy in the literature concerning the translocation mechanisms of cell-penetrating peptides, recent data suggests more than one mechanism. Studies using Tat-nanoparticles or Tat-liposomes suggest that the translocation mechanism occurs via energy-dependent macropinocytosis [43] and [49], whereas peptide-conjugated small molecules penetrate via electrostatic interactions and do not seem to depend on energy [50]. The exact mechanism of protamine-mediated penetration of microparticles into non-phagocytic cells remains to be clarified.

Taken together, we have shown the increased immunogenicity of antigens encapsulated in protamine-coated PLGA microparticles as compared to uncoated particles and proposed a possible mechanism of action. Further research to characterise protamine-coated particles is required to develop further a new technology combining the advantages of microparticles for prolonged antigen release and protamine for facilitating cell penetration of the delivery system. The potential applications of such an intracellular delivery system technology may span the areas of vaccine development, cancer immunotherapy, and gene/protein delivery.

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